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## Characterization of the *Lactococcus lactis* *pepN* gene encoding an aminopeptidase homologous to mammalian aminopeptidase N

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The nucleotide sequence of the *pepN* gene from *Lactococcus lactis* encoding a zinc-metallo aminopeptidase has been determined. The open reading frame of 2,538 base pairs encodes a protein with a calculated *M<sub>r</sub>* of 95,368, which agrees with the apparent *M<sub>r</sub>* of 95,000 of the gene product which was identified by polyclonal antibodies raised against the purified aminopeptidase. The amino acid sequence of the aminopeptidase of *L. lactis* was found to be similar to the corresponding enzymes of human, rat and mouse, with almost 30% of the residues identical. Also, a highly conserved area was identified which has similarity with the active site of thermolysin. A zinc-binding site, as well as the catalytic site for PepN, is predicted to lie within this conserved stretch. Putative promoter regions upstream of *PepN* were confirmed by primer extension analysis.

Aminopeptidase N; *Lactococcus lactis*; Mammalian

### 1. INTRODUCTION

Lactococci that are used in dairy fermentation are extremely fastidious and need an external source of amino acids. In order to grow on milk lactococci contain an efficient and specific proteolytic system which is composed of a cell envelope-associated proteinase and a number of peptidases [1]. The joint action of proteolytic and peptidolytic activities results in the degradation of casein into peptides and amino acids, which can then be taken up by the organism [2]. The purification and characterization of a dipeptidase [3], a tripeptidase [4], a prolidase [5], an X-prolyl-dipeptidyl-aminopeptidase [6], a glutamylaminopeptidase [7], an endopeptidase [8] and a general aminopeptidase (PepC) [9] have been reported. Recently, a 95-kDa aminopeptidase with a broad substrate specificity and a high activity has been purified from *L. lactis* subsp. *cremoris* Wg2 [10]. This aminopeptidase, which is found in all *L. lactis* strains studied [11], can hydrolyse several peptides derived from  $\beta$ -casein [12]. These observations indicate that the enzyme is an important component of the proteolytic system.

Until now, only the gene encoding the X-prolyl-dipeptidyl-aminopeptidase [13] from lactococci has been cloned and sequenced. This enzyme, which belongs

to the class of serine peptidases, shows no significant amino acid sequence similarity with other proteins. Also, no signal sequence was found for this enzyme.

Recently, the cloning, characterization and over-expression in *Escherichia coli* of the *L. lactis* gene for the 95-kDa general aminopeptidase has been reported [14]. The gene has been designated *pepN* since it complements an *E. coli pepN* mutation.

Further investigations reported in the present paper resulted in the complete DNA sequence of the *L. lactis* aminopeptidase N (3.4.11.2) and revealed extensive amino acid sequence homology with other peptidases.

### 2. MATERIALS AND METHODS.

#### 2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids are listed in Table 1. *L. lactis* was grown in M17 medium (Difco, East Molesey, UK) containing 1% lactose at 30°C. *Escherichia coli* was grown in TY broth (Difco, Detroit, MI) at 37°C with shaking. Chloramphenicol (10  $\mu$ g/ml), ampicillin (50  $\mu$ g/ml) or carbenicillin (50  $\mu$ g/ml) was added to the media when needed. When the pBluescript IKS<sup>+</sup> plasmid was used 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (0.01% (wt./vol.); Sigma Chemical Co., St. Louis, MO) and isopropyl- $\beta$ -D-thiogalactopyranoside (0.004% (wt./vol.)) (Sigma) was added to the agar medium. The polyclonal antiserum raised against the purified aminopeptidase from *L. lactis* subsp. *cremoris* Wg2, as well as the immunoblotting of the cell extracts, have been described by Tan et al. [11].

#### 2.2. Transformation and DNA manipulations

*E. coli* strains were transformed by the calcium chloride-rubidium chloride method, and general procedures for cloning and DNA manipulations were essentially performed as described [17]. DNA transfer to Genescreen Plus filters (NEN) followed by hybridization was performed according to specifications of the manufacturer. *L. lactis*

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MG1363 was transformed by electroporation using a high frequency transformation protocol. Restriction enzymes, All-For-One buffer and T4 DNA ligase were used as recommended by the manufacturer (Pharmacia LKB Biotechnology, Sweden).

### 2.3. DNA subcloning and sequencing

pNZ1101 is a plasmid derived from pACYC184 and contains an 8.2 kb *Pst*I fragment on which the *pepN* gene is located [14]. A 6.2 kb *Bam*HI-*Pst*I fragment was isolated from pNZ1101 [14] and subcloned into a pBluescript II KS<sup>+</sup> (pKS<sup>+</sup>) vector (Stratagene, La Jolla, CA, USA) yielding pTT01 (Fig. 1). *E. coli* JM101 was transformed with pTT01 and the expression of *pepN* was observed by immunoblot detection with polyclonal antibodies raised against the purified 95 kDa aminopeptidase from *L. lactis* subsp. *cremoris* Wg2 [10,11]. Unidirectional deletions into the DNA insert of pTT01 were made by *Exo*III/Mung Bean treatment (*Exo*III/Mung Bean Nuclease kit from Stratagene, La Jolla, CA, USA). pTT01 was first digested with *Bam*HI and *Sac*I in order to create appropriate deletions for sequencing the region downstream of the open reading frame (ORF). For sequencing the region upstream of the ORF, pTT01 was digested with *Cl*aI and *A*puI. Subsequently, the deleted pTT01 derivatives were transformed into competent JM101. After the transformants were grown on TY agar containing 50 µg/ml carbenicillin, plasmid DNA was isolated from the cells by the alkaline lysis procedure [18]. Clones containing appropriate overlapping inserts were identified by restriction analysis with

*Eco*RI and *H*indIII, and subsequently, prepared for DNA sequence analysis. Double-strand DNA sequencing was carried out for both strands by using [<sup>35</sup>S]dATP, the T7 primer (3' GATATCACTCAG-CATAA 5') and the reverse primer (5' AACAGCTATGACCATG 3'), and employing the dideoxy-chain termination procedure [19]. Some parts of the DNA were sequenced with the use of site-specific oligonucleotide primers. Oligonucleotides were synthesized with an automatic DNA synthesizer (Applied Biosystems model 381A). Computer alignment of the sequenced DNA fragment of both strands were carried out with the PC-gene software programme.

### 2.4. Enzyme activity in crude cell extracts

Cell-free extracts of *L. lactis* and *E. coli* were prepared and aminopeptidase N activity was determined as described previously [10]. Protein concentrations were measured by the method of Lowry et al. [20].

### 2.5. Primer extension experiments

Total RNA was isolated from cells of *L. lactis* MG1363 that had been converted into protoplasts as described previously [21]. Primer extension was performed by annealing 0.25 pmol of oligonucleotide (5' TATAATTTTCCGGAAC-3', complementary to the *pepN* gene, position 354–369, Fig. 3) to 15 µg of RNA followed by cDNA synthesis using reverse transcriptase as described [21]. Primer-extended products were separated on a 6% polyacrylamide/8 M urea sequencing gel

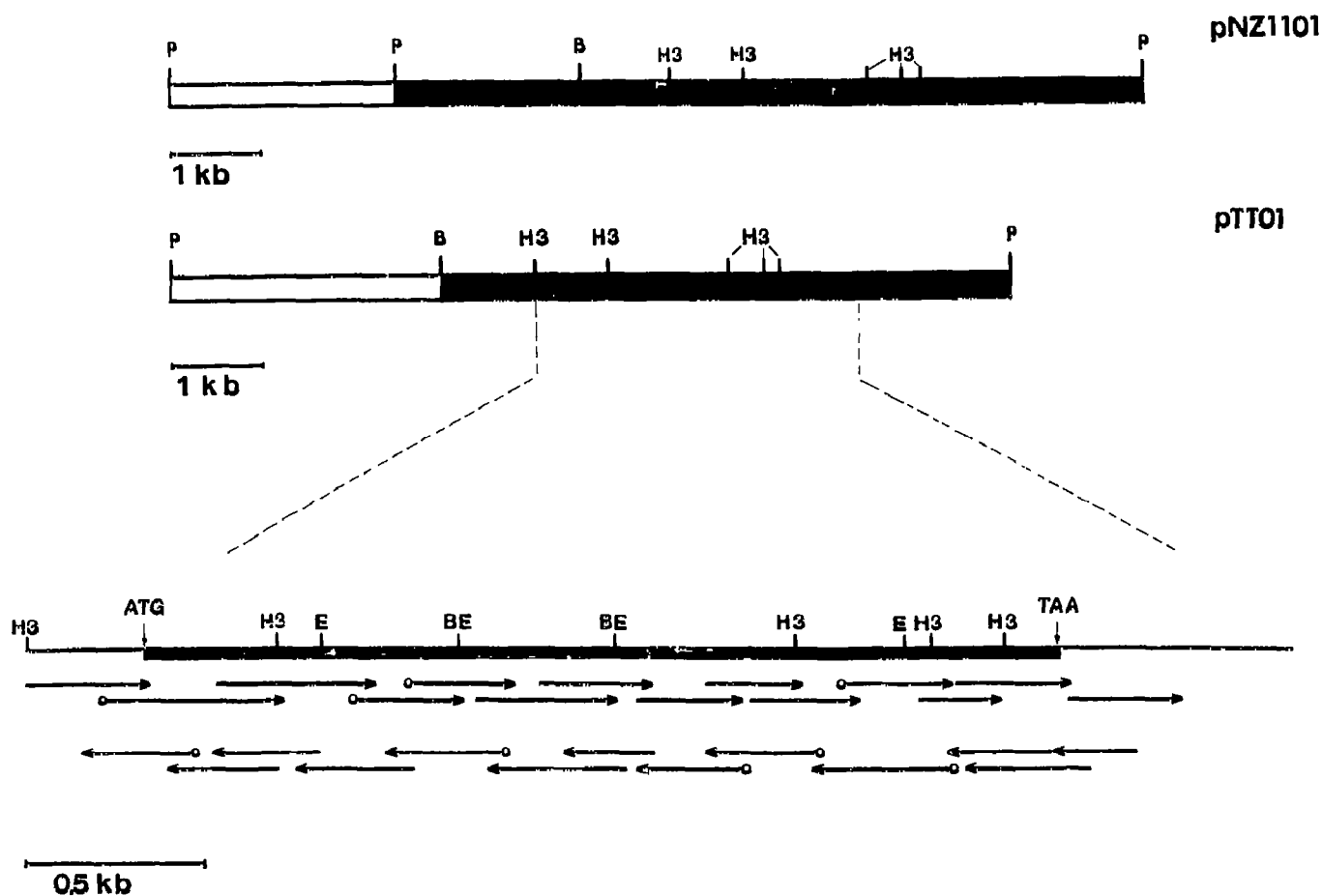


Fig. 1. Restriction endonuclease maps of two different *pepN*<sup>+</sup>-containing plasmids, pNZ1101 and pTT01 and the sequence strategy for pTT01. The black boxes indicate the *pepN* gene-containing fragments. Vertical lines indicate the cleavage sites of the following endonucleases, B, *Bam*HI; BE, *Bst*EII; E, *Eco*RI; H3, *H*indIII; P, *Pst*I. Vertical arrows indicate the beginning of the ORF (2,534 bp) starting with ATG and the end of the ORF with the stop codon, TAA. Horizontal arrows indicate the regions of both strands which were sequenced (see Materials and Methods).

Table I  
Bacterial strains and plasmids

Strains and plasmid	Relevant characteristics	Reference or source
<i>Strains</i>		
<i>E. coli</i> JM101	$\Delta$ ( <i>lac-proAB</i> ), [F', <i>lacI</i> <sup>u</sup> ZAM15]	Yanish et al. [16]
<i>L. lactis</i> subsp. <i>lactis</i> Mg 1363	Plasmid-free <i>pepN</i> <sup>+</sup>	Gasson [15]
<i>L. lactis</i> subsp. <i>cremoris</i> Wg2	<i>pepN</i> <sup>+</sup> strain	NIZO collection
<i>Plasmids</i>		
pNZ84	Cm <sup>r</sup> , pACYC184 derivative	Van Alen et al. [14]
pNZ1101	<i>pepN</i> <sup>+</sup> , pNZ84 derivative containing a 8.2 kb <i>Pst</i> I fragment	Van Alen et al. [14]
pBluescript IIKS <sup>+</sup>	Amp <sup>r</sup> , <i>lacZ</i> $\alpha$	Stratagene, La Jolla USA
pTT01	<i>pepN</i> <sup>+</sup> , pKS <sup>+</sup> derivative containing a 6.2 kb <i>Bam</i> HI/ <i>Pst</i> I fragment	This paper

*pepN*<sup>+</sup>, aminopeptidase N positive; Cm<sup>r</sup> and Amp<sup>r</sup>, resistance to chloramphenicol and ampicillin, respectively.

together with the products of a double-stranded sequence reaction obtained with the same primer and pNZ1104 DNA [14].

#### 2.6. Amino acid sequence comparison

A search of the NBRF/PIR (release 27.0) and SwissProt (release 17.0) databases was carried out in order to determine whether the *pepN* gene product shared sequence similarity with other proteins, through the facilities of the CAOS/CAMM Center, Nijmegen, The Netherlands. Multiple sequence alignment was performed using the program CLUSTAL [22].

### 3. RESULTS

#### 3.1. Subcloning and sequence analysis of the *L. lactis* *pepN* gene

A 6-kb *Bam*HI–*Pst*I fragment containing the *L. lactis* MG1363 *pepN* gene [14] was subcloned in the bluescript vector pKS<sup>+</sup> resulting in pTT01 (Fig. 1). The integrity of the cloned *pepN* gene was verified by analyzing *PepN* activity in *E. coli* JM101 harboring pTT01. The high lysylaminopeptidase activity (210 nmol/min/mg protein) obtained with the insert confirmed the previous conclusion [14] that expression is initiated within the cloned fragment. In addition, immunoblotting using polyclonal antibodies raised against purified aminopeptidase N [11] showed the presence of a single 95 kDa protein band in cell-free extracts of JM101 harboring pTT01 (Fig. 2).

The complete nucleotide sequence of the *pepN* gene

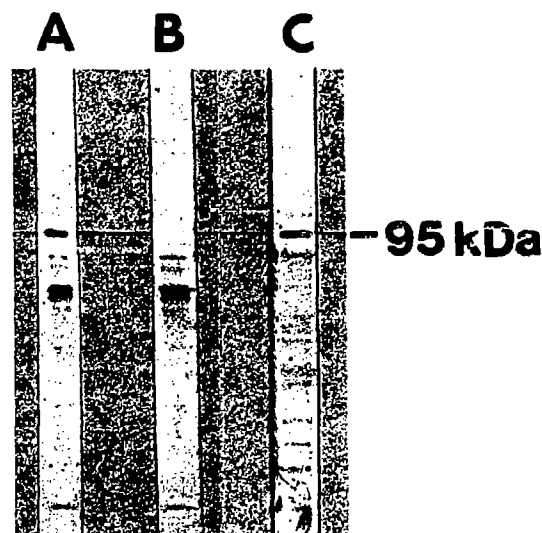


Fig. 2. Immunoblots of gels obtained by SDS-PAGE of cell-free extract from (A) *E. coli* JM101 harboring pTT01 (20  $\mu$ g of protein) (B) *E. coli* JM101 (20  $\mu$ g of protein) and (C) *L. lactis* subsp. *lactis* MG1363 (15  $\mu$ g of protein).

was determined (Fig. 3). A single open reading frame of 2,538 bp was found that starts with an ATG codon at position 324 and stops at the ochre termination codon (TAA) at position 2,862. The deduced protein has a size of 846 amino acids with a calculated  $M_r$  of 95,368, which corresponds well with that of the purified aminopeptidase N [10]. The N-terminal amino acids are identical to those reported for the purified aminopeptidase N from *L. lactis* Wg2 [10]. These amino acids are underlined in Fig. 3.

#### 3.2. Transcription initiation of the *pepN* gene

Primer extension experiments using RNA isolated from *L. lactis* MG1363 (Fig. 4) indicated that the major transcription initiation site of the *pepN* gene is located at the A residue at position 300. This site is preceded by canonical hexanucleotide sequences, TCGAAA and TATAAT, separated by 17 nucleotides that resemble the consensus sequences of lactococcal promoter elements [23]. Furthermore, the *pepN* gene promoter region contains a TG dinucleotide upstream from the –10 sequence that is conserved in various promoters from lactococci [23] and other Gram-positive bacteria [24]. The *pepN* gene is followed by an inverted repeat of 17 nucleotides that includes the termination codon (Fig. 3). If transcribed, this sequence may form a stem-loop structure with a  $\Delta G^0$  of –15.4 kcal/mol that may be involved in transcription termination [25]. If so, the *pepN* gene is a monocistronic transcriptional unit, which is in line with the observation that no open reading frames with a size greater than 50 bp are flanking the *pepN* gene (Fig. 3).





Fig. 4. Primer extension of RNA isolated from *L. lactis* MG1363. The relevant part of an autoradiographed standard sequencing gel is shown. Lanes 1–4 are A, G, C and T lanes, respectively, of a dideoxy sequencing reaction obtained with  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$  and unphosphorylated primer. Lanes 5 and 6 are extension products obtained with RNA and  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ , with and without the addition of unphosphorylated primer, respectively. Lane 7 is an extension product obtained with RNA and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  end-labeled primer. The slightly higher mobility of the product in lane 7 may be explained by the additional, negatively charged 5' phosphate group. The major transcription initiation site is indicated; the sequence shown is complementary to that presented in Fig. 3.

### 3.3. Amino acid sequence comparison

A protein database search revealed amino acid sequence similarity of the *L. lactis* aminopeptidase with the aminopeptidase N family (EC 3.4.11.2), including the zinc metalloenzymes from rat (965 residues) [26], human (967 residues) [27], rabbit (partial sequence, 791 residues) [28], pig (partial sequence, 294 residues) [29], mouse (945 residues) [30] and *E. coli* (870 residues) [31,32]. In addition, similarity was found with human leukotriene A4 hydrolase (LTA4H) (611 residues) [33], a zinc metalloenzyme with both epoxide hydrolase and peptidase activity [34]. A multiple sequence alignment of these enzymes is shown in Fig. 5. The highest overall similarity of the *L. lactis* aminopeptidase is found with mammalian aminopeptidases N (about 27% identical residues in the C-terminal 750 residues), while the overall similarity is considerably lower with aminopeptidase N from *E. coli* and human leukotriene A4 hydrolase. Six regions with highest amino acid identity are outlined in the multiple sequence alignment (Fig. 5). There is no significant similarity to any of the other enzymes N-terminal to the first boxed region, while C-terminal to the last boxed region there is significant similarity only to the mammalian aminopeptidases N. The hydropathy profile [35] of the aminopeptidase N from *L. lactis* did not reveal any hydrophobic stretches that could form a signal peptide or a transmembrane segment (not shown).

## 4. DISCUSSION

*E. coli* JM101 harboring pTT01 contained a 95-kDa protein, which could be detected with polyclonal antibodies raised against the purified aminopeptidase N from *L. lactis* subsp. *cremoris* Wg2 [11].

*L. lactis* aminopeptidase is found to belong to the aminopeptidase N family (EC 3.4.11.2). A multiple sequence alignment of the known members of this family shows that the best conserved segment, in which about 80% of the residues are identical, lies between residues 380 and 420 (this numbering is for rat aminopeptidase N; 281–301 are the equivalent residues in *L. lactis* (Fig. 5)). This highly conserved segment is also found in the thermolysin family of Zn-dependent neutral proteases [36] (not shown). From the three-dimensional structure

Fig. 3. Nucleotide and deduced amino acid sequence of pepN. Nucleotide residues are numbered in the 5'-to-3' direction, with the first A of AAGCTT of the *Hind*III restriction site numbered 1. The restriction sites as shown in Fig. 1 are indicated. The underlined amino acid sequence was also determined by protein sequence analysis of the purified enzyme. A putative ribosome-binding site is indicated by SD (Shine-Dalgarno) and by overlining. The putative -10 and -35 promoter regions are discontinuously underlined. The transcriptional starting point (+1), as determined by primer extension, is indicated by a vertical arrow. The TAA stop codon is indicated with stop. The shaded boxes indicate an inverted repeat with a  $\Delta G^0$  of -15.4 kcal/mol.

	1	50	100
AMPHRAT	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
AMPHUMAN	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
AMPHRABBIT	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
AMPHFIC	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
AMPHMOUSE	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
AMPHLACT	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
AMPHNECOLI	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
LTA4H	MAKGFYISKTLGILGILGVAACVCTIIALSVYVYAEKRNHAEISALNPLFSGS	SATT-STTNPAIDESKPNHNYRLPTLLPDSYQVTLAPYLTNPBQGLYIFKGSSTVRPTCNETTNVILHSHK	
	150	200	250
AMPHRAT	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
AMPHUMAN	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
AMPHRABBIT	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
AMPHFIC	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
AMPHMOUSE	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
AMPHLACT	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
AMPHNECOLI	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
LTA4H	KLNYTHKGNHVALRALGCTPAPNIDTTELVERTEYLVLHGLSGLKQV---	YEMDSEFQELADDLACFYRSEYMEGKNKRVVATT---FMQAADARKSFPCFDEPAKAFNITLILPHNL---TALSNMLPK	
	300	350	
AMPHRAT	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
AMPHUMAN	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
AMPHRABBIT	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
AMPHFIC	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
AMPHMOUSE	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
AMPHLACT	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
AMPHNECOLI	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
LTA4H	DSRT-LQDPSNWNVTNPHFPMSTYLLAYVSEFVYKVSFNRVQ---	IRIWARPSALDEGHODYALVYQFILNFAQHYNTAFLEKSDQIALP-DEKACAMENGLVYTRRESALVDFQSSSISNKER	
	400	450	500
AMPHRAT	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
AMPHUMAN	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
AMPHRABBIT	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
AMPHMOUSE	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
AMPHLACT	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
AMPHNECOLI	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
LTA4H	VVTVIAHELALQWQNLVTVVDMNDLWLNDFASVYVYADYAEPTNKLKDLVLNDVYRMAVDALASSHPLSSPANENYNTAQISELFD-BITTSKQASVLRMLSSFLT-EDLFRKQSSYLHTFQYSHI		
	550	600	
AMPHRAT	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
AMPHUMAN	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
AMPHRABBIT	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
AMPHMOUSE	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
AMPHLACT	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
AMPHNECOLI	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
LTA4H	YLDLNEHLQAVDSQATKLPASVSTMDRNLQMCFFVITVNTSTGCIYQEH-FLDPTKFRPSDFNYLHVIPIYLRNCK-EDHYWLET-ERNQSAEFQTS-NEVLLNINVTQYQYVYDENWARI		
	650	700	750
AMPHRAT	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
AMPHUMAN	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
AMPHRABBIT	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
AMPHMOUSE	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
AMPHLACT	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
AMPHNECOLI	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
LTA4H	QNLQQLDLSVIVPINAQIINDAFNLASACKLSITLPLNTLFLASETYMPEALSSLSYFK-LMDRSEVYCPM-KRYLKKQVTPLEAYFKIRTNHNLDRPP-TLMEQYNEINASTACSSGL-SECRDLV		
	800	850	
AMPHRAT	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
AMPHUMAN	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
AMPHRABBIT	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
AMPHMOUSE	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
AMPHLACT	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
AMPHNECOLI	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
LTA4H	VOLYSQMNNNSDNNFI-HPNLRSTVYCNALAQOGESE-WFAHEQFRATLVHEADKRLSALACSENVHILNRYLSYTLNPDYIRKQ---DATSTIVSIANNHYGQTLVYDFVRSNHRK---LPEDYGGGSFS		
	900	950	
AMPHRAT	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		
AMPHUMAN	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		
AMPHRABBIT	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		
AMPHMOUSE	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		
AMPHLACT	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		
AMPHNECOLI	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		
LTA4H	FANLQGVTRFSEFF-ELQQLQEFKEDNSATGOSOTRALBQALERTANKHVKENKDVYKHTENS		

Fig. 5.

of thermolysin from *Bacillus* [37] and elastase from *Pseudomonas* [38] it is known that this segment is part of the active site and contains an essential Zn ion binding site. The sequence identity in the highly conserved segment suggests that in *L. lactis* aminopeptidase N the residues His-288, His-292 and Glu-311 are Zn ion ligands and that Glu-289 is involved in catalysis.

The multiple sequence alignment shows that the sequence of the first 40 residues of the aminopeptidase N from human, rat, pig and mouse contain the typical characteristics of a signal sequence [39]. These sequences include a hydrophobic stretch of over 20 residues which presumably functions as a membrane anchor. The mammalian aminopeptidase N's are known to be cell surface ectoenzymes [26,29]. In contrast, the aminopeptidase N's from *L. lactis* and *E. coli* and the LTA4 hydrolase are considerably shorter at the N-terminus and lack such a hydrophobic stretch. While the *L. lactis* and *E. coli* enzymes are approximately of the same size (namely 846 and 870 residues, respectively), they seem to have diverged in evolution to the extent that sequence homology is significant in less than half of the molecule, i.e. only in the domain between residues 200 and 560 (using AMNPRAT. numbering (Fig. 5)).

Most oligopeptides generated from  $\beta$ -casein by the proteinase are probably not transported across the cytoplasmic membrane. The presence of a di/tri-peptide uptake system which has a high affinity for Leu-Pro [40], as well as the presence of amino acid transport systems [41] in *L. lactis*, implies that at least 3 extracellular proteolytic enzymes are needed: a general aminopeptidase [10], an X-prolyldipeptidyl-aminopeptidase [6] and a glutamylaminopeptidase [7]. However, localisation studies [11] showed that aminopeptidase N [10], as well as aminopeptidase C [9], are present intracellularly but that the X-prolyl-dipeptidyl-aminopeptidase [6] is found in the cell envelope. The intracellular location of the aminopeptidase N from *L. lactis* subsp. *cremoris* Wg2 and aminopeptidase C from *L. lactis* subsp. *cremoris* AM2 ([9,10], Chapot-Chartier, pers. commun.) are consistent with the lack of hydrophobic stretches likely to be a signal sequences in these enzymes.

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Fig. 5. Computer alignment of the amino acid sequence of the PepN protein from *L. lactis* (AMPN<sub>LACT</sub>) with those of other PepN proteins from rat (AMPN<sub>RAT</sub>) [26], human (AMPN<sub>HUMAN</sub>) [27], rabbit (AMPN<sub>RABBIT</sub>) [28], pig (AMPN<sub>PIG</sub>) [29], mouse (AMPN<sub>MOUSE</sub>) [30], *E. coli* (AMPN<sub>COLI</sub>) [31,32] and human leukotriene A<sub>4</sub> hydrolase (LTA4H) [33]. Identical (\*) amino acids in the sequences are indicated. The most conserved areas are indicated with boxes. The signal sequences of PepN from rat, human, pig and mouse are underlined. The putative zinc-binding residues and residues required for catalytic activity are indicated by Z and C, respectively. The numbering shown corresponds to that of AMNPRAT.



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